Human Adipogenesis Assay Kit
PPAR γ Agonists

Cat# DIF-AG; DIF-AG-NC

INSTRUCTION MANUAL ZBM0005.05

STORAGE CONDITIONS

Frozen subcutaneous preadipocytes

Store in liquid nitrogen IMMEDIATELY upon receipt. No expiration date is applicable; however, the cells must be plated within 1 week of receiving the kit to account for the expiration of the kit components.

Media, Reagents A & B, Buffers:

Store at 2 - 8°C. **Use reconstituted Glycerol Reagent A within 7 days.**

Glycerol Standard

-20°C

ORDERING INFORMATION AND TECHNICAL SERVICES

- **Zen-Bio, Inc.**
- **3200 Chapel Hill-Nelson Blvd., Suite 104**
- **PO Box 13888**
- **Research Triangle Park, NC 27709**
- **Telephone** (919) 547-0692
- **Facsimile (FAX)** (919) 547-0693
- **Toll Free** **1-866-ADIPOSE (866)-234-7673**
- **Electronic mail (e-mail)** **information@zen-bio.com**
- **World Wide Web** **http://www.zenbio.com**

INTRODUCTION

The differentiation assay kits provide the tools to study the compounds that stimulate cultured human adipocyte differentiation or lipogenesis. Such compounds may be PPARγ agonists or a combination of thiazolidinediones and glucocorticoids that are potentially useful in the treatment of diabetes.

This kit is designed to test compounds as potential PPARγ agonists. It is our experience that the PPARγ agonist used as the Positive Control sufficiently stimulates human adipocyte differentiation after 7 days of treatment. The protocol is designed so that the test compounds are also used in a 7-day treatment regimen. This kit contains sufficient reagents to assay 100 assay points in a 96 well format.

ITEMS INCLUDED IN THE KIT

Item	Description	Unit	QTY	Item	Storage
Human SQ Preadipocytes	Human subcutaneous preadipocytes, ≥ 2.0 X10^6 cells/vial, cryopreserved	VIAL	1	1	Liquid nitrogen
PM-1	Preadipocyte medium (See Appendix A)	BOTTLE	50ml	2	4°C
NC	Negative control: (See Appendix A)	VIAL	500 µl	2	4°C
PC	Positive control: (See Appendix A)	VIAL	500 µl	2	4°C
VC	Vehicle control (See Appendix A)	VIAL	500 µl	2	4°C
IM	Initiation medium (See Appendix A)	BOTTLE	300ml	3	4°C
MM	Maintenance medium (See Appendix A)	BOTTLE	100ml	2	4°C
Wash buffer		BOTTLE	50ml	2	4°C
Lysis buffer		BOTTLE	15ml	2	4°C
Glycerol Reagent A	Reconstitute w/ 11.0 ml deionized water prior to use. **Use reconstituted reagent within 7 days.**	BOTTLE	11.0ml	2	4°C
Reagent B	Reconstitute w/ 2.5 ml deionized water prior to use	BOTTLE	2.5 ml	2	4°C
Glycerol standard	Glycerol @ 1mM [Reconstitute with 400 µl Standards Diluent to make the 200 µM glycerol standard; see page 5 for recommended dilution scheme]	VIAL	100 µl	2	-20°C
Standards diluent		BOTTLE	2 ml		4°C
Tray	Clear polyvinyl tray for multi-channel pipetters	EACH	3	-----	-----
Data sheet	Certificate of Analysis and protocol	EACH	1	-----	-----
Assay Plates,blank	96-well assay plate, blank	PLATE	3	---	-----

Other equipment/reagents required but not provided with the kit:

- Additional Maintenance Medium, if necessary (see background information)
- Single-channel pipetter
- Multi-channel pipetter
- Plate reader with a filter of 540 nm
- Tubes to dilute glycerol standards

ASSAY PROCEDURE

A. DIFFERENTIATION PROCEDURE

On each day of the procedure, the appropriate medium must be warmed to 37° C prior to use.

Note: This protocol is designed to accommodate a weekday work schedule if started on a Monday-Thursday. Any deviation may require weekend work. We strongly recommend testing all compounds in triplicate.

Day 1: This is the day the cells are plated.

1. Remove cells from liquid nitrogen and place immediately into a 37° C water bath and agitate while in bath. Be careful not to submerge the cap of the vial into water. Do not leave the vials in water bath after most of the content has thawed. Rinse the vials with 70% ethanol before taking them to the culture hood.

2. Upon thawing, transfer the cells to a sterile conical bottom centrifuge tube containing 10 ml of Preadipocyte medium (cat # PM-1).

3. Centrifuge: 1,200 rpm (282Xg) / 20°C / 5 minutes. Aspirate the supernatant. TAKE CARE TO NOT ASPIRATE ANY OF THE CELL PELLET.

4. The cell vial contains a minimum of 2.0 x 10^6 viable cells; however, we recommend performing a cell count to determine a more exact number of cells. Resuspend the cell pellet in 2 ml Preadipocyte medium; dilute an aliquot in 0.4% trypan blue solution. We suggest withdrawing an aliquot of 50 μl of cells and mixing with 100 μl of the trypan blue solution, resulting in a dilution factor of 3. Count live (unstained) cells on a hemacytometer. The cell concentration required for approximately 40,000 cells / cm^2 in the 96 well format with 150μl /well is 1.3 x 10^6 cells in 15 ml Preadipocyte medium.

5. Plate cells in one of the 96 well plates provided in the kit. Do not agitate the plate, as cells will not plate evenly.

6. Place plate in 37°C incubator, 5% CO_2, 97% humidity. The cells will be maintained in the incubator after each manipulation until Day 14.

NOTE: This kit contains a sufficient volume of Initiation medium (IM) to use 10 ml of medium per compound dilution for a maximum of 29 compounds tested in triplicate (87 wells) on the 96-well plate, leaving 9 wells for controls. If a compound stock is too concentrated to accomplish the desired dilution, use an appropriate solution (not

supplied) to prepare an intermediate concentration that would allow for a final volume of 10 ml.

Also the positive control in this kit, the PPARγ agonist, has a final solvent concentration of 0.1% DMSO. This concentration is included in the vehicle control (VC). If the concentration of any solvent for the compounds used is high enough to potentially alter differentiation, please include that solvent concentration as an additional treatment. We do not recommend treating the cells with solutions exceeding 1% of any solvent, as higher concentrations may be toxic to the cells.

Day 2:

7. Twenty-four hours later, check the cells for confluence.
8. Using the Initiation Medium (IM), prepare treatments. Plan to do all treatments in triplicate. A blank plate map is included in these instructions to record the well treatments.
9. When all treatments are prepared, remove Preadipocyte medium from control wells. We recommend doing the treatments in small groups so the cells do not dry out.
10. Pipet 150 μl Positive Control, (PC), 150 μl Negative Control (NC), and 150 μl Vehicle Control (VC) into appropriate wells.
11. Remove media from experimental wells and pipet 150 μl each treatment media into appropriate wells. Incubate the plate for 7 days.

Day 8:

12. Using a multichannel pipetter remove 90 μl media from all wells. Gently feed all wells with 120 μl of the Maintenance Medium (MM) that is provided with this kit. Incubate the plate for 7 days.

Day 15:

13. Cells are now mature. Proceed to part B.
14. The positive control wells should exhibit significantly greater lipid accumulation than the negative control wells or the vehicle control wells. Refer to page 9 for a picture of a typical positive control when the adipocytes are mature.

B. TRIGLYCERIDE ASSAY

1. Warm the Wash buffer and Lysis buffer in a 37°C water bath.

2. Prepare the Reagent B by adding 2.5ml deionized water per bottle and gently invert. DO NOT VORTEX! Use a pipet to ensure that the powder is completely dissolved. Keep at

room temperature. Store in a light protected bottle. Reconstituted Reagent B is stable for 60 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C). Bring Reagent B to room temperature.

3. Remove all media. Using about 15 ml of the wash buffer, wash the cells one time with 150 µl wash buffer. Label the disposable tray "wash buffer" and retain for later use.

4. Remove all Wash buffer. Using a new tray, add 15 µl Lysis buffer. Incubate at 37°C – 50°C for 20 minutes.

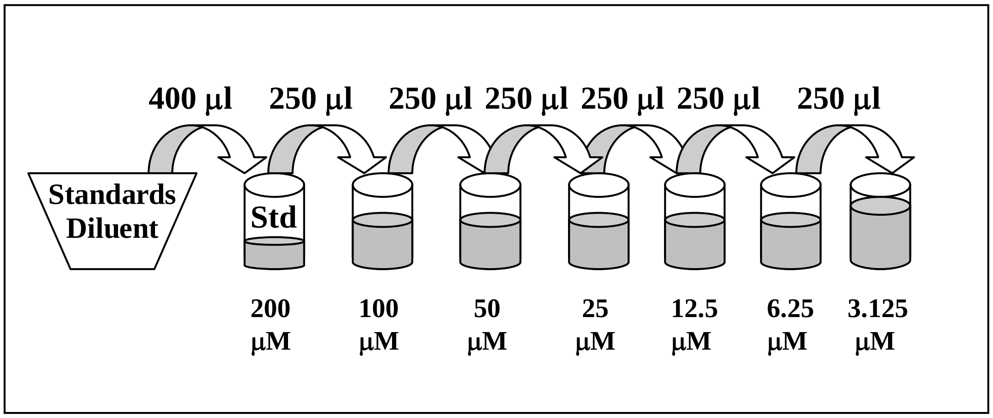
5. After the incubation is complete, visually confirm cell lysis by checking the wells under a microscope. If cells are not fully lysed, incubate another 10 minutes.

6. Add 135 µl warm Wash Buffer and mix the lysates by pipetting up and down three times.

7. Add 20 µl Reagent B to each well. It is not necessary to mix at this time, however, gently tap the plate to help mix the reagents. Incubate the plate at 37°C for 2 hours.

8. Bring Reagent A and the glycerol standards to room temperature during this time. The Wash Buffer can also be kept at room temperature at this point. Warm the Standards Diluent to 37°C. Prepare the standard curve as follows:

   Pipette 400 µl of the Standards Diluent into the 1 mM glycerol standard tube provided and mix well by vortexing. This produces a diluted stock glycerol standard of 200 µM. Pipette 250 µl of diluent into 6 tubes (not provided). Using the newly diluted stock glycerol solution, prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The 200 µM stock dilution serves as the highest standard, and the diluent serves as the zero standard.



Note: The above dilution series generates enough volume to perform the standard curve in duplicate. If you wish to perform the standard curve in duplicate, please note that eight fewer data points can be assayed with this kit.

9. Also at this time prepare the Reagent A by adding 11.0 ml deionized water per bottle and gently invert. DO NOT VORTEX Use a pipet to ensure that the powder is completely dissolved. Keep at room temperature. If using a Reagent A solution previously prepared and stored at 2-8°C, also bring to room temperature. Make sure there is enough Reagent A from one solution to treat all the points in the assay. It may be necessary to combine solutions. Store in a light protected bottle. Reconstituted Glycerol Reagent A is stable for 7 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).

10. To a blank 96 well plate, add 80 μl wash buffer to each well needed for the assay (NOTE: do not add Wash Buffer to the wells used for the standard curve).

11. Working with one row or column at a time, mix the lysates very well using a multi-channel pipette. Immediately transfer 20 μl per well of the lysates to the corresponding well of the plate containing the wash buffer. **This results in a Dilution Factor of 5.**

12. Prepare the standard curve. Pipet 100 μl of each standard into a well. (NOTE: Eight wells are necessary for the curve or sixteen if performing the curve in duplicate. If there are remaining wells on the assay plate, you can utilize the remaining wells. If not, a second plate is included in this kit).

13. Using the third tray, add 100 μl Reagent A to samples and standards. Mix by pipetting up and down one time. Incubate at room temperature for 15 minutes.

14. Read at 540 nm using a microtiter plate reader.

GLYCEROL STANDARD CURVE __________________

This kit is designed to show relative lipid accumulation of experimental treatments compared to controls. The assay is based on the equation

1 M Triglyceride yields 1M glycerol + Free Fatty Acids

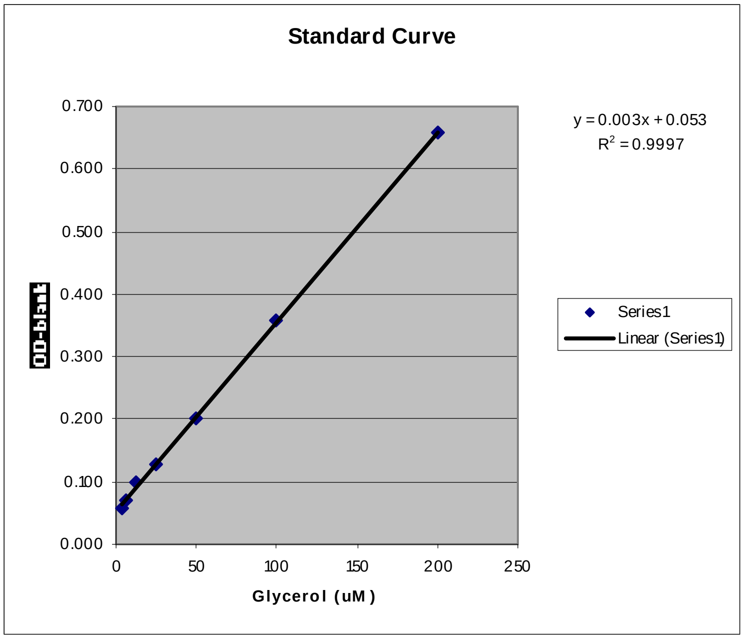
The reagent measures the concentration of glycerol released after lysing the cells and hydrolyzing the triglyceride molecules. The triglyceride concentration can then be determined from the glycerol values.

Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

- Subtract the OD value of the 0 μM standard from all OD values including the standard curve.

Glycerol (uM)	OD	OD	OD-blank	OD-blank	Avg OD-blank
0	0.048	0.048			0.048
3.125	0.059	0.058	0.011	0.01	0.059
6.25	0.07	0.07	0.022	0.022	0.070
12.5	0.098	0.098	0.05	0.05	0.098
25	0.127	0.13	0.079	0.082	0.129
50	0.2	0.205	0.152	0.157	0.203
100	0.353	0.362	0.305	0.314	0.358
200	0.649	0.667	0.601	0.619	0.658

slope	0.003
intercept	0.053
R^2	0.9997



y = observed O.D. minus the blank
x = concentration of glycerol in μM
To calculate x for each y, (i.e. to change the observed O.D. into glycerol concentration) use the following equation:
y=(slope) times (x) plus intercept
y=mx+b so x=(y-b)/m
x=(y – 0.0006)/0.0014 where 0.0014= slope of the line and 0.0006= y intercept.

Be careful to enter the proper sign for the y intercept value as it may be a negative number.
Any OD values greater than the highest standard (200 μM) should be suspect. The compound should be re-assayed using a lower dose of the compound at treatment OR a dilute solution of the condition medium at the time of the assay.

The R^2 value should be equal or greater then 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

Solve for the Total Glycerol concentration (i.e. total triglyceride concentration) for each OD. **Remember to include the Dilution Factor in the equation**. Data is expressed as μM Glycerol.

NOTE: Any OD values that are negative after the blank is subtracted should be considered to be 0 for the OD value.

TROUBLESHOOTING

Problem	Suggestions
High background or the triglyceride reagent turns a darker color before the assay begins.	Use clean tray and tips Change pipet tips frequently
Edge effects	Ensure a saturated humidity in the incubator to prevent evaporation from the outside wells
Inconsistent OD reading	Be careful when pipetting to avoid bubbles. If bubbles persist, burst the bubbles using a large gauge needle prior to reading and read the plate again.
Cells appear dead after 7 days treatment with my compound	An acute treatment for 3 days in Initiation Medium followed by additional feedings each 7 days should yield suitable positive control signal to complete the assay.

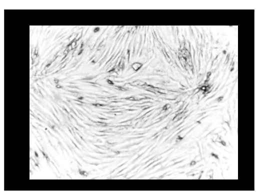
FREQUENTLY ASKED QUESTIONS

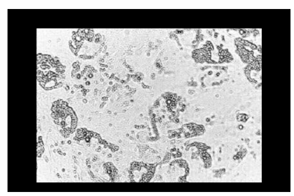
1. **Can I buy the reagents separately?** The only reagents sold separately are Glycerol Reagent A (cat# RGTA-10) and the glycerol standard for the Triglyceride Assay kit (cat# TG-GLYSTAN).
2. **Can I use another plate format besides 96 well?** This kit is designed for the assay of A 96 well plate (100 assay points). We do not have a protocol for other formats.
3. **Can I use this kit to measure total triglyceride in other cell lines and other human and non-human cells?** Yes. The assay is not species specific. As long as the sample concentration is in the linear range, this kit should be able to detect it.
4. **My cells did not lyse.** What can I do? If cells are not fully lysed, incubate for another 10 minutes at 37°C - 50°C. Sometimes mixing by pipetting up and down several times is necessary for full lysis.

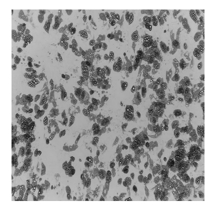
5. **I do not have time to complete the assay. Can I freeze the samples?** Yes. The cell lysates can be stored at -80°C for a maximum of 7 days. Mix the thawed lysates in the plate by pipetting up and down several times. Allow all reagents and samples to reach room temperature BEFORE adding the Wash Buffer and Glycerol Reagent A to complete the assay.

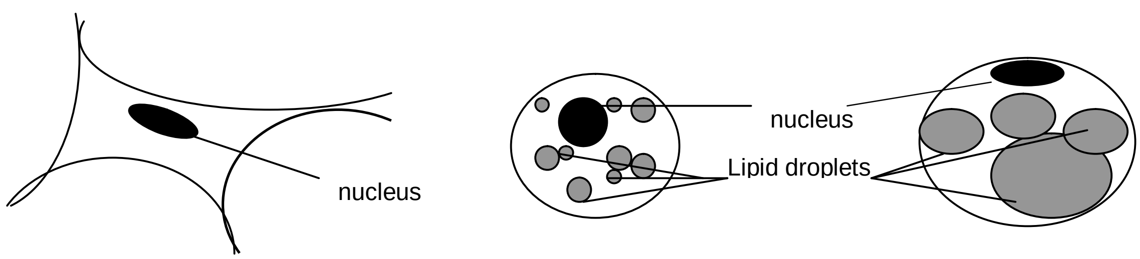
APPENDIX A. DIFFERENTIATION PICTURES

PREADIPOCYTE ——————————————→MATURE ADIPOCYTE









APPENDIX B: COMPOSITIONS OF KIT REAGENTS

Reagents	**Vehicle Control (VC)** **IBMX, 0.1% DMSO**	**Negative Control (NC)** **IBMX, PPARγ agonist, TNFα**	**Positive Control (PC)** **IBMX, PPARγ agonist**
Components	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • DMSO	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • PPARγ agonist • TNFα	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • PPARγ agonist
Reagents	**Preadipocyte Medium**	**Maintenance Medium (MM)**	**Initiation Medium (IM)**
Components	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Penicillin • Streptomycin • Amphotericin B	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX)

APPENDIX C: PLATE LAYOUT ______________________

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

APPENDIX D: DIFFERENTIATION FLOWCHART ______

DAY 1

PLATE CELLS. INCUBATE 24 HOURS 37°C

↓

DAY 2

APPLY TREATMENTS IN INITIATION MEDIUM

INCUBATE 7 DAYS 37°C

↓

DAY 8

CHANGE CELLS TO MAINTENANCE MEDIUM

INCUBATE 7 DAYS 37°C

↓

DAY 15

CELLS ARE MATURE. MOVE ON TO

TRIGLYCERIDE ASSAY PROTOCOL

APPENDIX E: TRIGLYCERIDE ASSAY FLOWCHART__

Remove all media from wells.

↓

Wash with 150 µl Wash Buffer
Remove all Wash Buffer from wells and add 15 µl Lysis Buffer.

↓

Incubate 20 minutes at 37 – 50°C

↓

Verify lysis and add 135 µl warm Wash Buffer. Mix by pipetting up and down 3 times.

↓

Add 20 µl Reagent B and incubate 2 hours at 37 °C.

↓

One hour prior to assay, reconstitute Glycerol Reagent A and prepare standards. Keep all at room temperature.

↓

Add 80 µl Wash Buffer to a new plate. Mix lysates and transfer 20 µl lysates to the wells containing Wash Buffer.

↓

Transfer 100 µl of each standard to a new plate.

↓

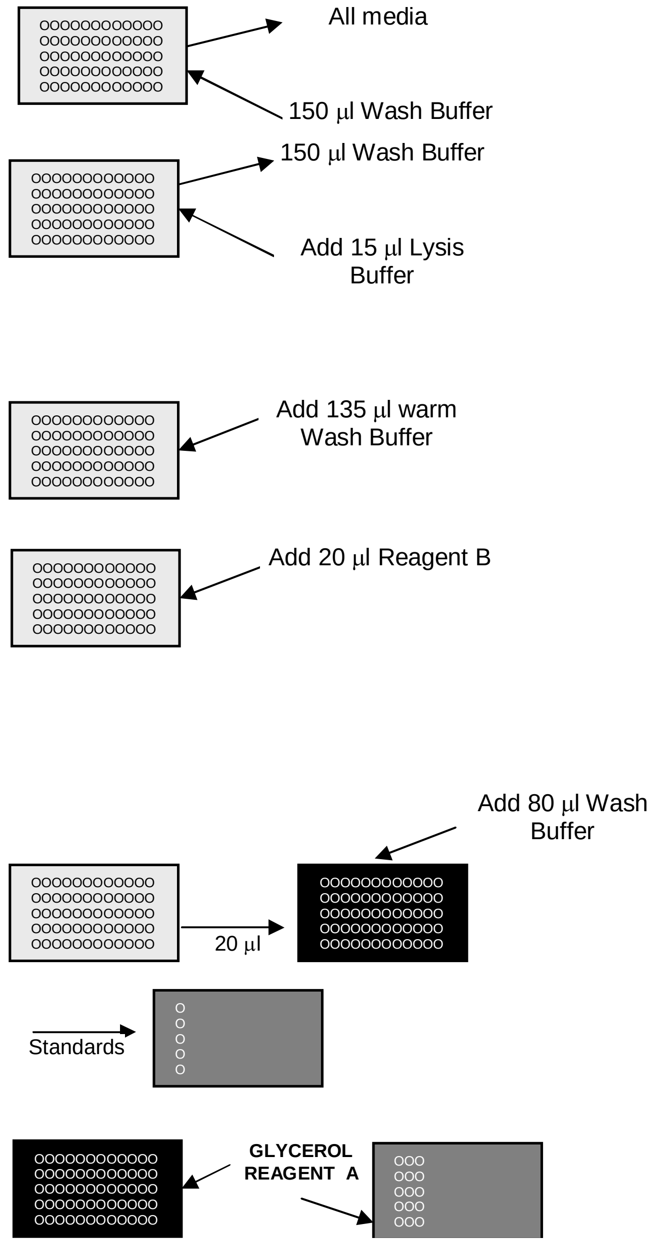
Add 100 µl Glycerol Reagent A to samples and standards.

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Incubate 15 minutes at 25°C (room temperature). Pop the bubbles in each well.

↓

Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.



An additional blank assay plate may be necessary for the assay of glycerol standards.